have a cholate backbone with polar groups extending from one face and a short alkyl chain extending from the opposite face. FA-solubilized Cx26 crystallized into the *H*32 space group with two monomers in the asymmetric unit, and the crystals diffracted isotropically to 3.3-Å resolution. Using a molecular replacement search model based on a cryoEM map of Cx43 at 5.7-Å resolution [Fleishman *et al.*, *Mol. Cell* 15: 879-888 (2004)], we solved the structure independently from a previously reported 3.5-Å resolution X-ray structure [2ZW3, Maeda *et al.*, *Nature* 458: 597-602 (2009)]. The overall R/Rfree values and completeness were 0.311/0.328 and 98.9%, respectively, and the Molprobity score was 2.07 (100th percentile). The RMS differences between 2ZW3 and our structure were 0.9 and 1.7-Å for the main-chain and side-chain atoms in the TM helices and 1.3 and 1.9-Å for the main-chain and side-chain atoms in E1 and E2. Although the topology and fold recapitulated 2ZW3, the maximum differences were significant: 2.7 and 5.9-Å for the main chain and side chain atoms in the TM helices and 3.9 and 7.6-Å for the main-chain and side-chain atoms in E1 and E2. We generated an electron density map at a resolution comparable to the cryoEM structure of the authentic Cx43 channel. The similarity of the maps suggests that detergent-solubilized Cx26 that crystallized as a dodecamer represents the authentic gap junction channel.

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Hydrogen Bonds at the Docking Interface are Critical for Functional Gap Junction Channel Formation of Cx26 and Cx32

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Gap junctions are unique intercellular channels formed by proper docking of two hemichannels from adjacent cells; each hemichannel is a hexamer of connexins - the gap junction proteins encoded by 21 homologous genes in human genome. Docking of two hemichannels to form a functional gap junction channel is only possible between the hemichannels formed by compatible connexins. The underlying docking mechanism for compatibility is not clear. Based on the crystal structure of Cx26 gap junction channel, we developed homology structural models and generated a series of mutants on extracellular domain 2 (E2). According to the model, 36 hydrogen bonds (HBs) were indentified at E2-E2 interface between a pair of Cx32/Cx26 hemichannels. The HB-forming residues between E2 domains are conserved in Cx32, Cx26 and heterotypically compatible connexins, but not in non-compatible connexins. Asp175 of Cx32 was a pivotal residue forming 3 HBs with K168, T177 and D179 of inter-docked Cx26. Cx32 mutations, N175Y or N175H, destroy 3/3 or 2/3 HBs, respectively, at the E2-E2 docking interface. Experimentally, these two mutants failed to form putative gap junction plaques and were unable to form homotypic functional gap junction channels. Morphological and functional tests of various combinations of these mutants with designed mutant and wild-type Cx26 revealed that the hydrogen bonds at the E2 docking interface are critical for docking compatibility in the gap junctions formed by Cx26 and/or Cx32. Restoring more hydrogen bonds at the docking interface was able to rescue the function of Cx32N175H, but not N175Y using designed mutant or wild-type Cx26. Our results demonstrate that HBs at the E2-E2 docking interface are key factors for heterotypic docking compatibility between Cx32 and Cx26 hemichannels and possibly other hemichannels formed by compatible connexins.

Platform: Membrane Physical Chemistry II

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Bilayer Asymmetry, Cholesterol Content, and Ligand Binding Influence Membrane Protein Sequestering in Raft-Mimicking Lipid Mixtures

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Lipid heterogeneities and membrane proteins are widely considered to show an important functional relationship in plasma membranes. However, the analysis of this intriguing relationship is challenging, due to the small size and transient nature of lipid heterogeneities in the plasma membrane environment. Recently, we presented a powerful model membrane platform that allows the thorough analysis of membrane protein sequestering and oligomerization in well-defined heterogeneous lipid environments using confocal fluorescence intensity analysis paired with a photon counting histogram (PCH) method (1). By applying this experimental approach, here we show that bilayer asymmetry has a significant influence on the sequestering of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Our experiments demonstrate a higher affinity of integrins for the liquid-disordered (l_d) phase in the presence of bilayer-spanning liquid-ordered (l_o)-l_d phase separations. In contrast, a preference for the l_o phase is observed in an asymmetric bilayer with l_o-l_d phase separations, which are exclusively located in the top leaflet of the bilayer. Importantly, PCH analysis shows that the observed changes in integrin sequestering are not caused by altered receptor oligomerization states. In another set of experiments, we also demonstrate that changes in cholesterol may have a profound impact on integrin sequestering without altering receptor oligomerization state. The obtained results are discussed in terms of potential changes in lipid packing density and hydrophobic thickness within the model membrane. The experimental model membrane approach is also applied to explore the functionally important relationship between the sequestering, level of dimerization, and ligand binding of the GPI-anchored urokinase receptor.

(1) Siegel, A. P. et al. (2011) *Biophys J***101**(7): 1642-1650.

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Steric Pressure between Proteins Opposes Membrane Phase Separation

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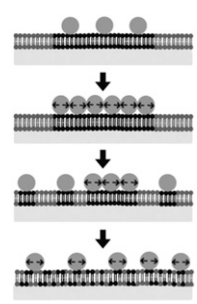
From endocytosis to intercellular signaling, biological processes require precise and rapid assembly of protein complexes on membrane surfaces. How is this accomplished within the complex and crowded environment of cellular membranes? Lipid rafts, defined as phase-separated lipid domains enriched in cholesterol and saturated lipids, are thought to locally organize specific proteins, assisting the assembly of protein complexes. However, the extent to which lipid rafts can concentrate proteins has not been experimentally measured. Using a reconstituted system, we varied the density of proteins bound to the surfaces of lipid domains. Surprisingly, we found that when membrane-bound proteins became crowded, steric pressure arising from collisions between proteins destabilized lipid domains (Figure). These results demonstrate that protein-protein steric pressure creates a significant energetic barrier to the stability of phase-separated biological membranes. Comparison with a simple analytical model reveals that domains are destabilized when steric pressure exceeds the approximate enthalpy of membrane mixing, a threshold that larger membrane-bound proteins reach more efficiently. These results provide a new perspective on the role of lipid rafts as organizers of membrane proteins and suggest that a dynamic balance of membrane surface pressures governs the stability of phase-separated cellular membranes.



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Controlling Bilayer Curvature and Membrane Protein Density using Droplet Interface Bilayers

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A Droplet Interface Bilayer (DIB) forms when nanolitre aqueous droplets are contacted together in an oil solution in the presence of phospholipids: A lipid monolayer forms at each oil-water interface, and by bringing together two monolayers a bilayer is created.

We have recently extended this methodology to modulate both two-dimensional protein concentration[1] and membrane curvature.

Manipulation of the axial position of the droplet relative to a hydrogel substrate controls the size of the bilayer formed at the interface; this enables the surface density of integral membrane proteins to be controlled. We are able to modulate the surface density of integral membrane proteins over a range of 4 orders of magnitude within a timeframe of a few seconds. By imaging DIBs on curved substrates we have observed correlation of lipid domain formation with sites of intrinsic curvature. This technique provides a new method for dictating the curvature of artificial bilayers, enabling single molecule measurements on the role of curvature in membrane organization and function.

[1] Gross LCM, Castell OK, Wallace MI. Nano Letters (2011) http://dx.doi.org/10.1021/nl201689v

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Studying Bending Rigidity of Model Vesicles and Cell Plasma Membrane using Lipid Nanotubes

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Mechanical properties of artificial lipid membranes have been examined by variety of techniques, like X-ray diffraction, NMR, micropipette aspiration or fluctuation analyses. However, new methods able to study membranes in most natural environment and with potential to approach cell membranes directly are desirable.